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Nitric Oxide: Physiology, Pathophysiology, and Pharmacology

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I. Introduction

THE demonstration in 1987 of the formation of NO* by an enzyme in vascular endothelial cells opened up what can now be considered a new area of biological research (for review, see Moncada et al., 1989). NO, which accounts for the biological properties of EDRF, is the endogenous stimulator of the soluble guanylate cy-

*Abbreviations: NO, nitric oxide; EDRF, endothelium-derived relaxing factor; ACh, acetylcholine; Hb, hemoglobin; GMP, guanosine monophosphate; SOD, superoxide dismutase; L-NMMA, N^G-monomethyl-L-arginine; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); GTN, glyceryl trinitrate; L-NA, N^G-nitro-L-arginine; L-NIO, N-iminoethyl-L-ornithine; L-NAME, N^G-nitro-L-arginine methyl ester; SNP, sodium nitroprusside; ADP, adenosine diphosphate; NMDA, N-methyl-D-aspartate; NANC, nonadrenergic, noncholinergic; LPS, lipopolysaccharide; IFN-γ, interferon-γ; TNF, tumour necrosis factor; FMLP, formyl-methionyl-leucyl-phenylalanine.

clase. In addition, NO is an effector molecule released by murine macrophages and other cells after immunological activation.

NO is synthesized from the amino acid L-arginine by an enzyme, the NO synthase. In the last year it has become apparent that there are at least two types of this enzyme. One is constitutive, cytosolic, Ca^{2+}/calmodulin dependent, and releases NO for short periods in response to receptor or physical stimulation. The NO released by this enzyme acts as a transduction mechanism underlying several physiological responses. The other enzyme is induced after activation of macrophages, endothelial cells, and a number of other cells by cytokines and, once expressed, synthesizes NO for long periods. Furthermore, this enzyme is cytosolic, Ca^{2+} independent, it requires tetrahydrobiopterin as well as other cofactors, and its induction is inhibited by glucocorticoids. So far, the only clearly established role for this NO is as a cytotoxic molecule for invading microorganisms and tumour cells. It is likely, however, that the release of NO via this enzyme has other biological consequences including pathological vasodilation and tissue damage.

This review will consequently be divided into two major areas, one relating to the role of NO as a transduction mechanism and the other to its role as an effector molecule whose release is induced during immunological reactions.

II. Nitric Oxide as a Transduction Mechanism for the Soluble Guanylate Cyclase

A. Vasculature

1. Early observations of endothelium-dependent relaxation and endothelium-derived relaxing factor from 1980 to 1987. In 1980, Furchgott and Zawadzki demonstrated that the vascular relaxation induced by ACh was dependent on the presence of the endothelium and provided evidence that this effect was mediated by a labile humoral factor, later known as EDRF. Endothelium-dependent relaxation, which was subsequently demonstrated in many vascular preparations, including some veins, arteries, and microvessels, occurs in response to a variety of substances, such as ACh, adenine nucleotides, thrombin, substance P, the calcium ionophore A23187, and bradykinin. Other stimuli, such as hypoxia, increase in flow, and electrical stimulation, also cause endothelium-dependent relaxation of vascular tissue in vitro. Some agents, however, such as the nitrovasodilators, atrial natriuretic factor, bovine retractor penis inhibitory factor, β-adrenergic agonists, and prostacyclin, induce vascular relaxation by endothelium-independent mechanisms (for reviews, see Furchgott, 1984; Griffith et al., 1984; Busse et al., 1985; Moncada et al., 1986b).

The humoral nature of EDRF was first demonstrated using a variety of pharmacological preparations in which the biologically active substance was transferred from a donor to a detector bioassay. One such system consisted of a "sandwich" arrangement of two rabbit aortic strips in which the EDRF donor (a strip with intact endothelium) was placed, intimal surface to intimal surface, next to the detector (a strip without endothelium; Furchgott 1984). Another approach involved perfusion of the lumen of an intact rabbit isolated aorta, the effluent of which was used to superfuse endothelium-denuded vascular rings (Griffith et al., 1984; Rubanyi et al., 1985). Stimulation of the donor aorta with ACh caused relaxation of the detector tissues. Finally, vascular endothelial cells, cultured on microcarriers and packed in the barrel of a syringe or a modified chromatography column, were perfused, and the effluent was used to superfuse a ring of canine coronary artery or a series of rabbit aortic strips denuded of endothelium (Cocks et al., 1985; Gryglewski et al., 1986a).

It was established, using techniques such as these, that EDRF was a very short-lived substance with a half-life of only seconds in oxygenated physiological salt solutions (Griffith et al., 1984; Cocks et al., 1985). Release of EDRF was observed under basal conditions as well as after stimulation with ACh (Griffith et al., 1984; Rubanyi et al., 1985; Martin et al., 1985). The effects of EDRF were shown to be inhibited by Hb, methylene blue (Martin et al., 1985), and other agents such as dithiothreitol and hydroquinone (Griffith et al., 1984) and to be mediated by stimulation of the soluble guanylate cyclase with the consequent elevation of intracellular cyclic GMP levels (Rapoport and Murad, 1983).

Bioassay studies in which the source of EDRF, either fresh vascular tissue with endothelium (Rubanyi et al., 1985) or vascular endothelial cells in culture (Cocks et al., 1985; Gryglewski et al., 1986a), was separated from the detector (endothelium-denuded vascular rings or strips) allowed the study of the effects of physical or chemical manipulation on the generation, stability, or actions of EDRF. It was found using such techniques that superoxide anions (O_2^-) contribute to the instability of EDRF, because the effects of EDRF were prolonged by the addition of SOD (Gryglewski et al., 1986b; Rubanyi and Vanhoutte, 1986) and inhibited by Fe^{2+} (Gryglewski et al., 1986b) and hyperoxia (Rubanyi and Vanhoutte, 1986). Furthermore, a number of compounds described as inhibitors of EDRF were shown to act by generating O_2^- in solution as a result of their redox properties. Indeed, SOD attenuated their inhibitory effects on the action of EDRF (Moncada et al., 1986a). These observations led to the prediction and subsequent confirmation that another substance capable of removing O_2^-, cytochrome *c*, would attenuate the action of these redox compounds on EDRF and that an O_2^--generating compound, pyrogallol, would also act as an inhibitor (Moncada et al., 1986a). This latter compound has now been used by a number of authors to investigate the biological properties of EDRF (Ignarro et al., 1987; Matsunaga and Furchgott, 1989; Shultz and Raij, 1989).

Generation of O_2^-, however, does not account for the action of all inhibitors of EDRF, because Hb does not act by this mechanism (Martin et al., 1986; Hutchinson et al., 1987).

EDRF was also shown to inhibit platelet aggregation (Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987a), to cause disaggregation of aggregated platelets, and to synergize with prostacyclin in both of these actions (Radomski et al., 1987b). In addition, EDRF inhibits platelet adhesion to endothelial monolayers, extracellular matrix, and collagen fibrils (Radomski et al., 1987c,d).

2. Identification of endothelium-derived relaxing factor as nitric oxide. Early suggestions that EDRF might be a product of the arachidonic acid lipoxygenase (Singer and Peach, 1983; Forstermann and Neufang, 1984) or of the cytochrome P-450 enzyme system (Pinto et al., 1985; Macdonald et al., 1986) or was a compound with a carbonyl group near its active site (Griffith et al., 1984) did not lead to the identification of its chemical structure. Based on the similarities in the pharmacological behaviour of EDRF and NO generated from acidified NO_2^-, Furchgott suggested in 1986 that EDRF may be NO (see Furchgott, 1988). At the same time, Ignarro et al. also speculated that it may be NO or a closely related species (see Ignarro et al., 1988).

The first evidence for the formation of NO by mammalian cells came from experiments in which EDRF released from vascular endothelial cells was detected by the chemical means used to identify NO. NO may be measured directly as the chemiluminescent product of its reaction with ozone (Downes et al., 1976). It was shown using this method that the concentrations of bradykinin that induced the release of EDRF from porcine aortic endothelial cells in culture also caused a concentration-dependent release of NO. Moreover, the amounts of NO released by the cells were sufficient to account for the relaxation of vascular strips (Palmer et al., 1987; fig. 1). Furthermore, the levels of NO released by these cells also accounted for the inhibition of platelet aggregation and adhesion induced by EDRF (Radomski et al., 1987b,c). A key element in all of these studies was the correlation between the amounts of NO measured by bioassay and those detected by chemiluminescence.

A detailed comparison of the biological actions of EDRF and NO on vascular strips (Palmer et al., 1987; Hutchinson et al., 1987) and on platelets (Radomski et al., 1987a) also showed that the two compounds were indistinguishable (Moncada et al., 1988b). Both EDRF and NO caused a relaxation of the vascular strips that declined at the same rate during passage down the bioassay cascade (Palmer et al., 1987; fig. 2). Furthermore, the rate of decay during transit in polypropylene tubes was slower but similar for both compounds, indicating that they have identical chemical stability even under these artificial conditions. Both EDRF and NO also inhibited

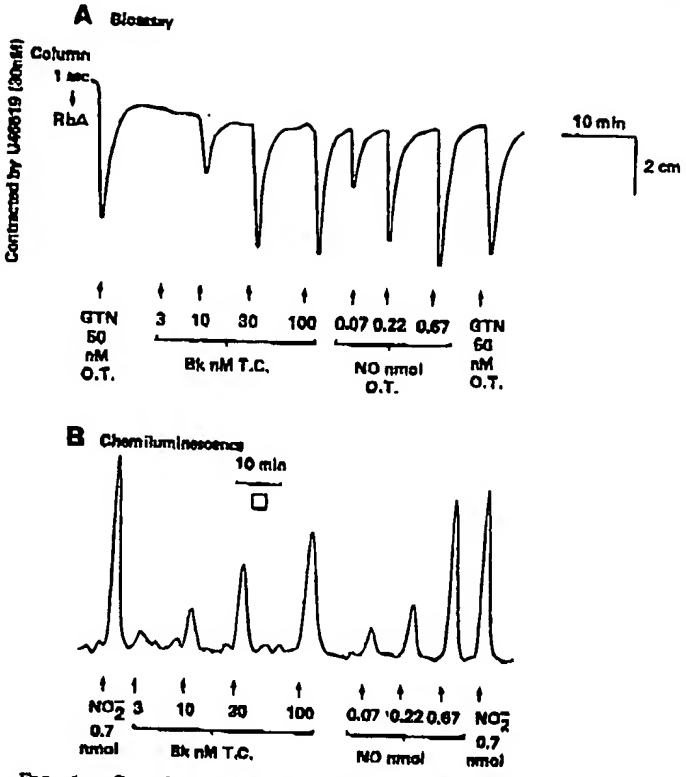

FIG. 1. Correlation between NO measured by bioassay and by chemiluminescence. *A*, Bioassay: relaxation of rabbit aorta by EDRF and NO. A column packed with endothelial cells cultured on microcarriers was perfused with Krebs' buffer (5 ml/min). The effluent was used to superfuse three spiral strips of rabbit aorta (RbA), denuded of endothelium, in a cascade (top tissue only shown in this figure). The tissues were contracted submaximally by a continuous infusion of 9,11-dideoxy-9α, 11α-methano epoxy-prostaglandin $F_{2\alpha}$ (U46619; 30 nM) and were separated from the cells by delays of 1, 4, and 7 s, respectively. The response of the detector tissues was calibrated by administration of a standard dose of GTN (50 nM) over the tissues (O.T.). The bioassay tissues were relaxed in a concentration-dependent manner by EDRF released from the cells by 1-min infusions through the column (T.C.) of bradykinin (Bk; 3 to 100 nM) and by NO (0.07 to 0.67 nmol, O.T.) dissolved in He-deoxygenated water and administered as 1-min infusions. *B*, Chemiluminescence: release of NO by bradykinin (Bk) from a replicate column of the cells used in the bioassay. The amounts of NO (administered as a 1-min infusion into the column effluent) which relaxed the bioassay tissues were also detectable by chemiluminescence. Effluent from the column, or Krebs' buffer into which authentic NO was injected, was passed continuously (5 ml/min) into a reaction vessel containing 75 ml 1.0% sodium iodide in glacial acetic acid under reflux. NO was removed from the refluxing mixture under reduced pressure in a stream of N_2, mixed with ozone, and the chemiluminescent product was measured with a photomultiplier. The amounts of NO detected were quantified with reference to a NO_2^- standard curve. □, area equivalent to 0.22 nmol NO. Reprinted with permission from Nature 327: 524–526, 1987 (Macmillan Magazines Ltd.).

platelet aggregation (Radomski et al., 1987a), induced the disaggregation of aggregated platelets (Radomski et al., 1987b), and inhibited platelet adhesion (Radomski et al., 1987c,d). Moreover, their biological half-lives as inhibitors of platelet aggregation were similar (Radomski et al., 1987a).

The actions of EDRF and NO on vascular strips and on platelets were similarly potentiated by SOD and cy-

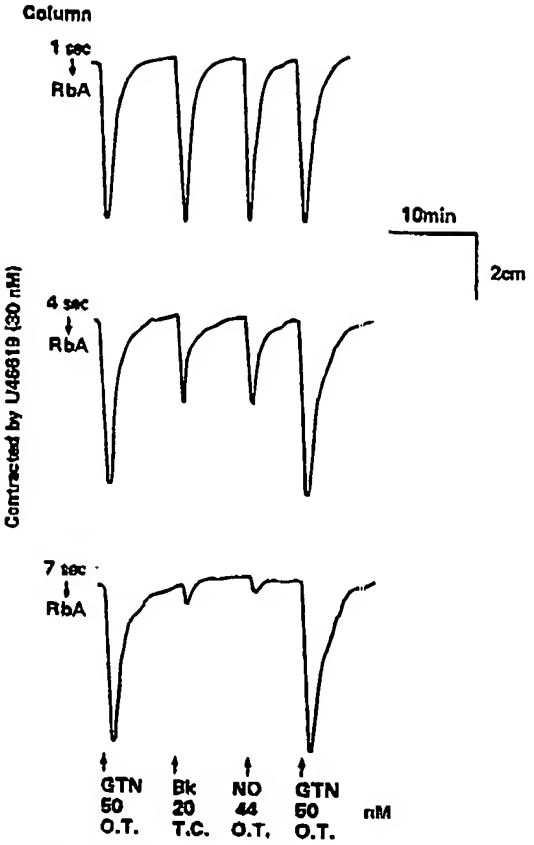

FIG. 2. Comparison of the stability of EDRF and NO during passage down a bioassay cascade. EDRF (released by bradykinin, 20 nM) and NO (44 nM) decay at similar rates. For details of experimental procedure, see legend to fig. 1A. Reprinted with permission from Nature 327: 524–526, 1987 (Macmillan Magazines Ltd.).

tochrome *c* and inhibited by Fe^{2+} and some redox compounds (Palmer et al., 1987; Hutchinson et al., 1987; Radomski et al., 1987a). Furthermore, the potency of redox compounds as inhibitors of EDRF-induced and NO-induced vascular relaxation was attenuated by SOD to a similar extent. In addition, the inhibitory action of Hb on EDRF can be explained by the fact that this substance binds avidly to NO (Hermann, 1865; Gibson and Roughton, 1957; Martin et al., 1986). Finally, both EDRF and NO act on vascular smooth muscle (Kukovetz et al., 1979; Rapoport and Murad, 1983) and platelets (Mellion et al., 1981) through the stimulation of soluble guanylate cyclase and elevation of cyclic GMP.

NO release from vascular endothelial cells from other species and from a number of vascular preparations, in amounts sufficient to account for the biological actions of EDRF, has also been demonstrated. It was also shown, using a chemical assay based on the diazotization of sulfanilic acid by NO and subsequent coupling with N-(1-naphthyl)-ethylene diamine, that NO or a labile nitroso species was released from perfused bovine pulmonary artery. Furthermore, perfusion of segments of pulmonary artery or pulmonary vein with A23187 caused relaxant responses and elevation of vascular cyclic GMP levels in the bioassay tissues that could be matched by NO (Ignarro et al., 1987). Similar results were obtained in perfused rabbit aortae stimulated with ACh, A23187, and substance P (Khan and Furchgott, 1987; Chen et al., 1989). Later, the use of a spectrophotometric assay, based on the reaction between NO and Hb, also demonstrated the release of NO from vascular endothelial cells in culture (Kelm et al., 1988). Furthermore, the release of NO from isolated perfused rabbit (Amezcua et al., 1988) or guinea pig (Kelm and Schrader, 1988) hearts has been shown to account for the vasodilator actions of ACh and bradykinin in these preparations.

All of this evidence strongly supported the proposal that EDRF is NO. Moreover, NO fulfilled the criteria for identification as a biological mediator, as originally defined by Dale (1933).

3. Controversy about the chemical identity of endothelium-derived relaxing factor. Although the evidence for EDRF being NO is compelling, several lines of research have questioned this conclusion. These include observations about variations in the half-life of EDRF, differential binding of EDRF and NO to anion exchange columns, differential activity of EDRF and NO on smooth muscle preparations, stabilization of EDRF, and poor correlations between biological activity and the amounts of NO detectable by chemical methods.

Wide variations in the half-life of EDRF (from 3 to 50 s; Griffith et al., 1984; Forstermann et al., 1984; Rubanyi et al., 1985; Cocks et al., 1985; Gryglewski et al., 1986b) can probably be explained in terms of the relative contributions of O_2 and O_2^- to the inactivation of NO under different conditions. Oxygen is known to react rapidly with NO to form NO_2 which in solution forms nitrite (NO_2^-) and nitrate (NO_3^-), both of which are almost inactive on platelets and vascular strips (Radomski et al., 1987b; Palmer et al., 1987). NO has also been shown to react with O_2^- to form NO_3^- (Blough and Zafiriou, 1985). Thus, different half-lives are likely to be reported as the experimental conditions vary from laboratory to laboratory, making these studies of limited value. To date, no systematic study has been conducted of the chemistry of NO in physiological solutions at 37°C.

EDRF has been reported to bind to anion exchange columns (Cocks et al., 1985; Long et al., 1987), whereas NO does not (Long et al., 1987). However, other workers have shown that amounts of NO that induce comparable relaxation of the bioassay tissues also bind to these columns, although to a lesser extent (Khan and Furchgott, 1987; R. M. J. Palmer and S. Moncada, unpublished results). NO is not anionic, but it is known to nitrosate amines and, therefore, it is likely that the interaction of EDRF and NO with these columns is a chemical reaction.

The response of the bioassay tissues to bolus administration of an agonist differs quantitatively from that to an infusion. The release of NO after stimulation is likely to occur at variable rates for periods in excess of 1 min.

Because of this, the comparison between NO released from cells or tissues and bolus injections of NO may be misleading.

Some authors have reported that EDRF only relaxes vascular smooth muscle, whereas NO relaxes vascular, tracheal, and taenia coli smooth muscle (Shikano and Berkowitz, 1987; Dusting et al., 1988a). These results are difficult to analyse because neither group presented evidence showing that equieffective amounts of EDRF and NO were compared. The guinea pig tracheal strip is approximately 30 times less sensitive to infusions of NO than the rabbit aortic strip, suggesting that the amount of EDRF required for its relaxation is in excess of that released by porcine cells in culture (Dusting et al., 1988b; R. M. J. Palmer and S. Moncada, unpublished results). Furthermore, several laboratories have reported that, under appropriate experimental conditions, EDRF from cultured endothelial cells relaxes the same nonvascular smooth muscle preparations as does NO (Gillespie and Sheng, 1988; Angus and Cocks, 1989; Buga et al., 1989; Furchgott et al., 1990).

EDRF has been reported to be stabilized by acidification (Murray et al., 1986), a condition that would not be expected to stabilize NO. However, this can be explained in terms of the transformation of NO into NO_2^-, which can generate NO when acidified. In support of this, it has been shown that NO_2^- has the same chromatographic mobility as that reported (Murray et al., 1986) for the "stable form" of EDRF (R. M. J. Palmer and S. Moncada, unpublished results).

EDRF released from cultured bovine aortic endothelial cells by bradykinin has been found to be much more stable than NO when the effluent from which it was detected was immediately cooled to 0°C (Angus and Cocks, 1989). Furthermore, when the effluent was lyophilized and then reconstituted, it still had a major part of the initial relaxing activity, suggesting that EDRF may be a stabilized precursor of NO. An alterative explanation is that bradykinin stimulated the release from these cultured cells of both NO and a substance (or substances) that reacted with NO to form a much more stable product that can release NO on contact with tissue at 37°C. Such a product may be similar to the inhibitory factor from the bovine retractor penis (Gillespie and Sheng, 1988; Martin et al., 1988). The acid-activatable precursor of this factor is now thought to be NO_2^- (Furchgott, 1988; Martin et al., 1988), although the NO formed on acidification is stabilized even after neutralization by some substance (or substances) also present in the extract.

Recently, EDRF has been suggested to be an unstable nitroso compound, such as S-nitroso-cysteine, based on comparisons of its potency with that of NO on vascular strips and on the dissociation between measurements of NO release made by bioassay and chemiluminescence (Myers et al., 1990). However, the EC_{60} for NO on vascular strips in this study was significantly greater than that reported by other workers. Furthermore, contamination of NO with NO_2^-, which can occur in the preparation or use of these solutions (Furchgott, 1990), could account for discrepancies in the measurements of NO by bioassay and by chemiluminescence, which measures both NO and NO_2^-. Because these authors report identical stability of NO, EDRF, and S-nitroso-cysteine, it is more likely that differences in potency or in the amounts of NO measured are simply methodological.

Evidence from electron paramagnetic resonance spectroscopy has also recently questioned the identification of NO as EDRF (Rubanyi et al., 1990; Vedernikov et al., 1990). These measurements are based on the reaction between NO and Hb to form paramagnetic nitrosyl-Hb and show that NO, but not EDRF, forms such a species under apparently equivalent conditions determined by bioassay. However, the sensitivity of this method is limited and NO_2^- can also form nitrosyl-Hb under some conditions (Doyle et al., 1988). Because this method is based on a chemical reaction, it is essential to compare NO and EDRF under the same conditions.

The finding that EDRF and NO have identical chemical stability and quantitatively and qualitatively identical biological actions indicates that, if EDRF is released from the endothelial cells as an unstable precursor, it must break down completely within 1 s. Because cell membranes are readily permeable to NO, it is difficult to envisage a role for such a precursor, which may not easily penetrate cell membranes. However, if the existence of such an intermediate is proven, it will not detract from the fact that the biological effects of EDRF are mediated ultimately by NO. Distinction between these options is unlikely to be achieved by bioassay experiments because the responses of tissues to NO, or unstable NO generators, administered under different conditions, cannot be clearly distinguished. It is likely, therefore, that this question will only be resolved when the biochemical mechanism of the synthesis of NO is clarified.

The existence of mechanisms other than NO which play a role in endothelium-dependent relaxation cannot be excluded at present. For example, stimulation of the endothelium of some arteries by ACh results in a hyperpolarization of the adjacent smooth muscle cells which may contribute to their relaxation (Komori and Suzuki, 1987; Feletou and Vanhoutte, 1988; Brunet and Beny, 1989). This hyperpolarization has been attributed to the release of a factor, termed endothelium-derived hyperpolarizing factor (Feletou and Vanhoutte, 1988; Chen et al., 1988; Taylor and Weston, 1988). Although it has been reported that endothelium-derived hyperpolarizing factor differs from NO in that it is not influenced by Hb or methylene blue (Chen et al., 1988; Taylor and Weston, 1988; Chen and Suzuki, 1989), NO has recently been shown to cause hyperpolarization of some arteries (Tare et al., 1990). Further work is required to clarify the

functional significance of hyperpolarization of both endothelial and smooth muscle cells.

The existence of other endothelium-dependent vasodilator mediators would not be surprising, because mechanisms subserving a biological function are usually multiple. It is important to stress, however, that endothelium-dependent relaxations that are susceptible to inhibition by Hb and methylene blue, indicating the involvement of NO and cyclic GMP, have been demonstrated in many vascular preparations, whereas evidence for other mechanisms is scant.

4. Synthesis of nitric oxide. Early work ruled out the likelihood of compounds such as NO_2^-, NO_3^-, NH_3, and hydroxylamine being the source of NO (R. M. J. Palmer and S. Moncada, unpublished results). In 1988, however, the amino acid L-arginine was shown to be the precursor for the synthesis of NO by vascular endothelial cells. Endothelial cells, cultured in the absence of L-arginine for 24 h prior to the experiments, showed a decrease in the release of EDRF induced by bradykinin and A23187 which could be restored by L- but not D-arginine (Palmer et al., 1988a). Furthermore, this enhancement only occurred in the presence of the L-arginine infusion, suggesting that the formation of NO was dependent on free L-arginine alone.

Because these data provided only circumstantial evidence for L-arginine being the precursor for the synthesis of NO, definitive experiments were carried out using mass spectrometry and [^{15}N]L-arginine which demonstrated the formation of ^{15}NO from the terminal guanidino nitrogen atom(s) of L-arginine when the cells were stimulated with bradykinin (Palmer et al., 1988a; fig. 3). The formation of NO_2^- from L-arginine by endothelial cells was reported by others (Schmidt et al., 1988b). The conversion of L-arginine to NO is specific because a number of analogues of L-arginine, including its D-enantiomer, are not substrates. Furthermore, it was found that the release of NO from endothelial cells in culture could be inhibited in an enantiomerically specific manner by L-NMMA (Palmer et al., 1988b), an inhibitor of the generation of NO_2^- and NO_3^- and citrulline from L-arginine in macrophages (Hibbs et al., 1987a).

Endothelial homogenates form citrulline from L-arginine by a mechanism that is NADPH dependent and inhibited by L-NMMA (Palmer and Moncada, 1989). In endothelial cell cytosol, depleted of L-arginine by anion-exchange chromatography, there was an L-arginine-dependent increase in cyclic GMP which was also concentration dependent, required NADPH, and was accompanied by the formation of [^{3}H]citrulline from [^{3}H]arginine (Moncada and Palmer, 1990). Both the production of [^{3}H]citrulline and the increases in cyclic GMP were inhibited by L- but not D-NMMA. All of these data are consistent with NO and citrulline being coproducts of the same enzymatic reaction. In addition, the formation of [^{3}H]citrulline and the increase in cyclic GMP were

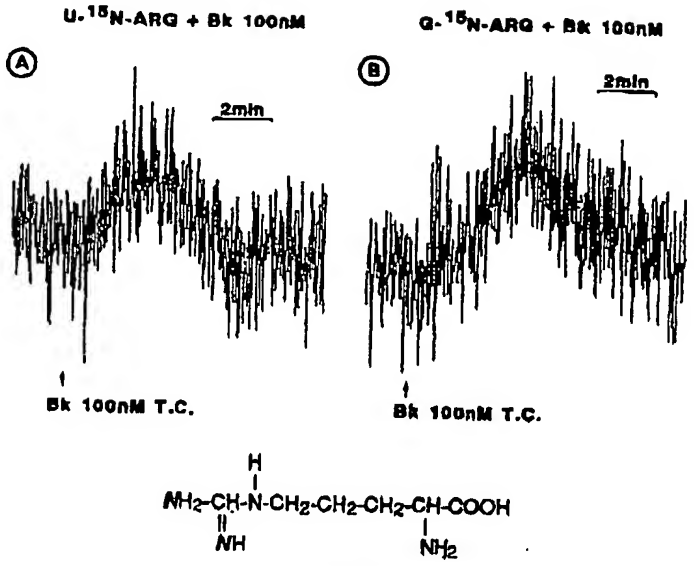

FIG. 3. Determination of ^{15}NO release from porcine aortic endothelial cells by mass spectrometry. A column packed with microcarriers covered with endothelial cells that had been cultured in medium without L-arginine (ARG) for 24 h was perfused with Krebs' buffer (5 ml/min). The effluent was passed continuously into the chemiluminescence reflux vessel, as described for fig. 1 except that NO was removed from the refluxing mixture under reduced pressure in a stream of He. The He stream was passed continuously into the source of an MS 50 TC (Kratos) mass spectrometer and ^{15}NO (m/z = 30.9950) was determined at high resolution by electron impact mass spectrometry with single ion monitoring. The identity of the signal was confirmed by reference to the natural abundance of ^{15}NO in NO standards which was found to be 0.37%. ^{15}NO was released from cells stimulated with bradykinin (100 nM) in the presence of an infusion of U^{15}N-arginine (10 μM; A) or G^{15}N-arginine (10 μM; B). The cells released 2.2 ± 0.4 nmol ^{15}NO (n = 3) in the presence of G^{15}N-arginine and 2.1 ± 0.7 nmol ^{15}NO (n = 4) in the presence of U^{15}N-arginine, indicating that NO is derived from a guanidino nitrogen atom of L-arginine (shown as *N*). Reprinted with permission from Nature 333: 664–666, 1988 (Macmillan Magazines Ltd.).

inhibited by Ca^{2+} chelators, indicating that this enzyme, which has now been called NO synthase, is Ca^{2+} dependent (Moncada and Palmer, 1990). Similar results have been reported by others (Mayer et al., 1989; Mulsch et al., 1989). Furthermore, NO synthesis from endothelial cell cytosol was inhibited by calmodulin-binding peptides and antagonists, an effect that was reversed by calmodulin, suggesting that the Ca^{2+}-dependent stimulation of NO synthase in endothelial cells is mediated by calmodulin (Busse and Mulsch, 1990a).

Recent evidence from studies using $^{18}O_2$ and mass spectrometry have shown that this enzyme incorporates molecular oxygen into both NO and citrulline, indicating that it is a dioxygenase (Leone et al., 1991).

5. Inhibition of the synthesis of nitric oxide in the cardiovascular system. a. IN VITRO. Data from experiments in vitro indicate that L-NMMA is a competitive inhibitor of the NO synthase (Palmer and Moncada, 1989; Mayer et al., 1989). An effect of this compound on

other arginine-metabolizing enzymes has not been reported, although it has been shown not to affect either arginase or arginine decarboxylase (Granger et al., 1990). L-NMMA also inhibits the release of NO from endothelial cells (Palmer et al., 1988b) and vascular tissues (Rees et al., 1989a; Amezcua et al., 1989). This compound has been a useful tool in the investigation of the biological significance of the L-arginine:NO pathway in the cardiovascular system.

L-NMMA induced an endothelium-dependent constriction of rabbit aortic rings, indicating that there is a continuous release of NO which maintains a dilator tone in this tissue (Palmer et al., 1988b). The removal of this basal tone accounts for all the constrictor activity of L-NMMA, including its apparent nonspecific endothelium-dependent vasoconstrictor effect on rat aortic rings (Thomas et al., 1989). Furthermore, L-NMMA inhibited endothelium-dependent relaxation induced by ACh, A23187, and substance P (Palmer et al., 1988b; Rees et al., 1989a). All of these effects could be reversed by L-arginine. Moreover, L-NMMA inhibited the release of NO induced by ACh from the perfused rabbit aorta, effects which were enantiomerically specific and reversible by L- but not D-arginine (Rees et al., 1989a). Inhibition by L-NMMA of histamine- and ACh-induced endothelium-dependent relaxation of the guinea pig pulmonary artery was also reversible by L-arginine (Sakuma et al., 1988). L-NMMA has since been used to examine the role of the L-arginine:NO pathway in the response to vasodilators in other vascular preparations in vitro, including rat aorta (Giuliani et al., 1990), canine cerebral arteries (Katusic et al., 1990), and human arteries and veins (Yang et al., 1990).

The importance of NO, formed from L-arginine, in regulating basal tone and the response to endothelium-dependent dilators in resistance vessels was demonstrated using L-NMMA in the isolated perfused rabbit heart (Amezcua et al., 1989). In this preparation, L-NMMA caused an increase in coronary perfusion pressure and an inhibition of the decrease in coronary perfusion pressure induced by ACh, accompanied by inhibition of the release of NO into the coronary effluent. These effects were enantiomerically specific and were attenuated by L- but not D-arginine. Interestingly, L-NMMA, by inducing what has been termed a transient, selective "biochemical denudation" of the preparation (Amezcua et al., 1989), revealed the direct vasoconstrictor action of ACh on smooth muscle. Some of these findings were subsequently observed in the isolated perfused guinea pig heart (Levi et al., 1990).

Recent evidence shows that removal of the endocardium induces a negative inotropic effect in isolated papillary muscle preparations (Smith et al., 1991). This, together with the finding that cultured porcine endocardial cells release NO and possess an NO synthase (Schulz et al., 1991), suggests that the L-arginine:NO pathway plays a role in myocardial contractility (Lewis et al., 1990; Smith et al., 1991).

b. IN VIVO. In anesthetized rabbits intravenous administration of L-NMMA, but not D-NMMA, induced an increase in blood pressure that could be reversed by L- but not D-arginine and was associated with a reduced release of NO from a perfused aortic segment obtained from treated animals (Rees et al., 1989b). This reduced release could be reversed by infusing L-arginine through the aortic segment in vitro. Thus, the basal release of NO derived from L-arginine plays an important role in regulating blood flow and pressure. Furthermore, the stimulated release of NO contributes to the response to endothelium-dependent vasodilators in vivo.

The hypotensive response to ACh in the anesthetized rabbit, rat, or guinea pig was inhibited only partially by L-NMMA (Rees et al., 1989b; 1990c; Whittle et al., 1989; Aisaka et al., 1990), and this effect was mainly detectable when expressed in terms of both the decrease and the duration of the hypotensive response. The reasons for this partial inhibition are not clear at present. Further work is required to clarify the mechanisms underlying these observations.

L-NMMA has subsequently been shown to cause an increase in blood pressure in anesthetized guinea pigs (Aisaka et al., 1989, 1990) and rats (Whittle et al., 1989; Gardiner et al., 1990a,b; Tolins and Raij, 1990; Tolins et al., 1990; Rees et al., 1990c). In one of these studies (Tolins et al., 1990), the vasodilation induced by ACh was accompanied by an increase in the urinary excretion of cyclic GMP, both of which were prevented by L-NMMA. Furthermore, the effects of L-NMMA on blood pressure were accompanied by a decrease in glomerular filtration rate.

The increase in blood pressure induced by L-NMMA was accompanied by a decrease in vascular conductance in the renal, mesenteric, carotid, and hindquarters vascular beds of conscious, chronically instrumented rats (Gardiner et al., 1990b). Furthermore, these effects were sustained if the infusion of L-NMMA was continued for 6 h (Gardiner et al., 1990c), indicating not only the critical role of NO in maintaining a dilator tone in all these beds but also the fact that regulatory systems in the vasculature are unable to reaccommodate the flow toward pretreatment levels. In awake, chronically instrumented dogs, L-NMMA induced a dose-related, L-arginine-reversible, constriction of the coronary circulation and a reduction in resting phasic coronary flow (Chu et al., 1991; fig. 4). The coronary vasodilation which follows vagal stimulation has also been suggested to be NO dependent (Broten et al., 1991).

Studies in which L-NMMA was infused in humans into the brachial artery or the dorsal veins of the hand demonstrated that the vasodilation induced by ACh or bradykinin, but not that induced by GTN, could be attenuated by this compound (Vallance et al., 1989a,b). Furthermore, whereas in the brachial artery L-NMMA induced direct vasoconstriction, it had no such direct